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Original Article

# Potential Effectiveness of Secondary Metabolites of *Azolla microphylla* as *Aedes aegypti* Repellent Guide

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## Abstract

Dengue Hemorrhagic Fever (DHF) remains an endemic public health concern, with *Aedes aegypti* mosquitoes serving as the primary transmission vector. While chemical repellents are widely used for vector control, their health and environmental risks have driven the need for safer, biodegradable natural alternatives. This study aimed to identify the secondary metabolite content of *Azolla microphylla* leaf extract and evaluate its effectiveness as a repellent against *Aedes aegypti*. This experimental study employed a post-test only control group design. Three extract concentrations (10%, 15%, and 20%) were tested alongside positive and negative controls, using 10 mosquitoes per group. Observations were conducted at 1, 2, 4, and 6 hours, with data analyzed using statistical tests to assess differences between treatment groups. Phytochemical screening confirmed the presence of flavonoids, terpenoids, and tannins in the extract. Repellent testing revealed that the 20% concentration provided the highest effectiveness, with an average mosquito repellency of 58% during the first 1–2 hours. However, effectiveness declined progressively across all concentrations over time. Statistical analysis confirmed a significant difference between treatment and control groups after 1 hour of observation ($p < 0.05$). *Azolla microphylla* leaf extract demonstrates potential as a natural repellent against *Aedes aegypti*; however, its protective effect is temporary due to the volatility of its active compounds. Further formulation development, such as encapsulation or the use of fixative agents, is needed to enhance the stability and durability of its repellent activity.

**Keywords**: *Aedes aegypti*, *Azolla microphylla*, Dengue Hemorrhagic Fever (DHF), Secondary Metabolites.

## 1. Introduction

*Aedes aegypti* is the main vector of Dengue Hemorrhagic Fever (DHF). DHF is caused by the dengue virus, transmitted by the female *Aedes aegypti* mosquito through its bite (Widiyaningtiyas et al., 2025; Wilke et al., 2021). The spread of DHF is often seen during the rainy season, because many puddles and pots or bathtubs serve as breeding grounds for *Aedes aegypti* mosquitoes (Adnyana et al., 2021). Female *Aedes aegypti* mosquitoes have the behavior of sucking blood repeatedly until their stomachs are full to meet their protein intake when the female mosquito lays eggs, thus increasing the efficiency of the spread of the DHF epidemic (Widiastara et al., 2022).

The spread of DHF is influenced by several factors, including temperature, mobility, population density, and community behavior, which are the foundation of efforts to prevent and control DHF (Terradas et al., 2024; Meier et al., 2022). According to the Ministry of Health, the prevalence of Dengue Hemorrhagic Fever (DHF) in Indonesia showed a significant increase from 2023 to 2024. In 2023, a total of 114,720 cases with 894 mortality were recorded, while in 2024, the cases jumped to 186,324 cases with 1,120 mortality (Dinas Kesehatan Provinsi Jawa Timur, 2024; Kemenkes, 2024). The level of presence and density of the mosquito population directly contributed to the spread of Dengue Hemorrhagic Fever in Indonesia.

Dengue Hemorrhagic Fever (DHF) control in Indonesia primarily relies on vaccination using the tetravalent dengue vaccine *(Dengvaxia)*, which protects against DENV1–DENV4. While proven safe and effective, its success depends on community participation alongside consistent mosquito control and environmental hygiene measures (Obembe & Oso, 2022; Felipe Oliveros-Díaz et al., 2022; Thomas, 2023). Control efforts include fumigation, nest eradication, larvicides, insecticides, and repellents (Rohmah et al., 2020). However, chemical repellents such as permethrin, malathion, and DEET pose health risks, cause environmental damage, and promote insecticide resistance (Irfayanti et al., 2022; Bayuadi et al., 2023; Patni et al., 2024), highlighting the need for natural, plant-based alternatives.

*Aedes aegypti*, the primary DHF vector, is influenced by temperature, rainfall, urbanization, and population mobility, with climate change further increasing transmission risk (Kraemer et al., 2015; Messina et al., 2019). Although chemical-based repellents and insecticides remain effective short-term (Maia & Moore, 2011), growing mosquito resistance and adverse health and environmental impacts raise serious concerns about their long-term sustainability (Shenashen et al., 2017; Yu et al., 2019).

Although interest in plant-based mosquito repellents is growing, existing research has largely focused on terrestrial plants, with limited attention given to aquatic plants such as *Azolla microphylla*. Studies on Azolla have demonstrated significant larvicidal activity and the presence of bioactive compounds including flavonoids, tannins, and saponins (Qian et al., 2018; Ravi et al., 2020), with further potential noted in nanotechnology-based bio-insecticide development (Wilson et al., 2023). Nevertheless, its effectiveness as a repellent against adult mosquitoes and the mechanisms by which it disrupts the mosquito olfactory system remain inadequately explored. This gap is both scientifically significant, as it limits the theoretical understanding of aquatic plant-based repellents, and practically significant, as communities remain heavily dependent on chemical repellents that pose health and environmental risks. Therefore, this study aims to fill that gap through the systematic exploration of *Azolla microphylla* as a natural, safe, and environmentally friendly repellent alternative.

Plants that have the potential to repel mosquitoes can also come from aquatic plants. One of them is Azolla, which has been studied by Ravi et al. (2018) and Ravi et al. (2020) on eggs, larvae and adult mosquitoes with high mortality rates. Azolla contains chemical compounds such as Diethyl Phthalate, 1-nonadecene, Neophytadiene, Hexadecanoic acid and methyl ester. Other research conducted by Ekawati & Pradana (2019) also shows that Azolla contains flavonoids, tannins and saponins. Research on Azolla as a natural mosquito repellent has not been widely conducted, so further research is needed to develop the potential of the Azolla plant. Mosquito repellents are currently not widely available, and research on natural ingredients is limited (Adnani et al., 2020). Excessive use of chemical insecticides can cause environmental pollution, so innovation is needed in the use of natural mosquito repellents that are safe for the environment. Therefore, this study aims to investigate the potential of *Azolla microphylla* as a natural mosquito repellent against adult *Aedes aegypti* mosquitoes.

# 2. Methods

## 2.1. Collection and preparation of *Azolla microphylla*

*Azolla microphylla* was taken from cultivators in Central Java. Azolla plants that have been obtained are then cleaned with running water and the extraction process is carried out.

## 2.2. Preparation of *Aedes aegypti*

*Aedes aegypti* mosquitoes were collected from the Provincial Health Office. The initial stage began with the hatching of mosquito eggs in chlorine-free water with a temperature of 25 to 30°C, pH 6.95 to 7.03 in the laboratory. Eggs that had hatched into larvae were then reared until they become pupae. The pupae were then transferred to mosquito cages to prevent the mosquitoes from escaping. Sucrose solution was provided in the mosquito cages as a food source for the mosquitoes (Patel et al., 2012).

## 2.3. Extraction of Secondary Metabolites

Extraction begins with the preparation of dry simplicia powder. The material was washed thoroughly with water then oven for 48 hours at 50°C then blended and sieved with mesh no. 20 (Macdonald et al., 2016; Nogata et al., 2006). Powder from the material was taken 2.5 kg and soaked with 6 L of n hexanes solution in a glass container and left for 48 hours while occasionally stirring, then filtered to obtain the residue and filtrate. The filtrate was evaporated and a thick hexane extract (non-polar) was obtained. The residue was re-soaked in 6 L of Ethyl acetate (EtOAc) solution for 48 hours, then filtered and the residue and filtrate were obtained. The filtrate was evaporated and a thick ethylacetate extract (semi polar) was obtained. The residue was re-soaked with 6 L of 70% ethanol (EtOH) for 48 hours and filtered to produce the residue and filtrate. The filtrate was evaporated and a thick ethanol condensed extract (polar) was obtained. The residue was re-soaked with a mixture of ethanol and DMSO in a ratio of 1:1 for 48 hours, then 1:1 for 48 hours, then filtered to produce the residue and filtrate. The filtrate was evaporated and obtained EtOH:DMSO (1:1, v/v) liquid extract (highly polar) (Ekawati et al., 2021); Adnani et al. (2020). Extract solution was then tested for secondary metabolites such as Flavonoid, Alkaloid, Saponin, Tanin and Terpenoid (Ekawati & Pradana, 2019; Larasati & Putri, 2023).

## 2.4. Preparation of Chili Fermentation

Chili solution is used because it emits heat like the human body (Mirmanto et al., 2017). Red chili peppers are blended as much as ½ kg until smooth then 500 ml of water is added and put into a closed container for 7 days. After 7 days the results of the marinade can be used (Prasetyo, 2021).

## 2.5. Mosquito Repellent Test

The study used 250 mosquitoes divided into 5 different cages measuring 30 × 30 × 30 cm, with treatment details as follows:

1) Treatment 1 (negative control): cup containing cotton swab, 2.5 ml chili fermentation water and 2.5 ml distilled water.
2) Treatment 2 (positive control): cup containing cotton, 2.5 ml of fermented chili water and 2.5 ml of DEET.
3) Treatment 3 (10% extract): cup containing cotton, 2.5 ml of fermented chili water and 2.5 ml of 10% *Azolla microphylla* extract.
4) Treatment 4 (15% extract): cup containing cotton, 2.5 ml of fermented chili water and 2.5 ml of 15% *Azolla microphylla* extract.

5) Treatment 5 (20% extract): cup containing cotton, 2.5 ml of fermented chili water and 2.5 ml of 20% *Azolla microphylla* extract.

Observation was made every 5 minutes at 0, 1, 2, 4 and 6 hours on mosquitoes perched on cotton wool in the cup. The results of the perching mosquito count were then calculated using the formula (Prasetyo, 2021):

$$\%Attached = \frac{B}{10} \times 100$$

### 2.6. Data Analysis

Data obtained were then presented in tabular form and analyzed using the Kruskal Wallis test using SPSS.

## 3. Results and Discussion

### 3.1. Phytochemical Test Screening

The results of secondary metabolite compound tests conducted showed that *Azolla microphylla* extract contains flavonoids, terpenoids, and tannins (Ekawati & Pradana, 2019). These three compounds are active compounds that can be used as mosquito repellents, especially terpenoid compounds that have a distinctive aroma that makes mosquitoes not want to approach or perch, thereby reducing the risk of bites, and have the ability to be toxic to the mosquito's body at high concentrations can cause mosquito death (Rashad, 2021). Flavonoid compounds have a mechanism of action that makes it difficult for mosquitoes to breathe, causing paralysis and triggering mosquito death. Meanwhile, tannin compounds can act as stomach poisons that can inhibit the digestive process and the enzyme system, causing death (Widiyaningtiyas et al., 2025).

### 3.2. Repellent Testing

This study used fermented chili peppers as a repellent testing medium to replace human hands and test animals. The use of fermented chili peppers is based on their ability to produce volatile organic compounds, including lactic acid and carbon dioxide, generated through the fermentation process, which act as olfactory attractants for Aedes aegypti mosquitoes, thereby mimicking the chemical cues typically associated with human skin (Mukabana et al., 2012; Smallegange et al., 2005). This method is considered safer, more consistent, and ethically sound as it eliminates the risk of skin irritation and reduces reliance on test animals. A fermented chili solution was applied by spraying it onto cotton, which also emits heat radiation approximating that of the human body, further enhancing its attractant properties (Adnani et al., 2020). However, it should be acknowledged as a methodological limitation that the degree to which fermented chili reliably replicates the full complexity of human skin odor has not been formally validated in standardized bioassay protocols. Consequently, the attractant intensity may differ from that of actual human skin, which could influence the absolute magnitude of mosquito landing rates. Future studies are encouraged to conduct comparative validation between fermented chili and human skin odor profiles to strengthen the reproducibility and standardization of this testing approach.

### 3.3. Effectiveness test of *Azolla microphylla* extract as a repellent

**Table 1. The Average Number of Mosquitoes that Land**

| Test Material (%) | Lots of samples (n) | The average number of mosquitoes that land | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 Hours | | 1 Hours | | 2 Hours | | 4 Hours | | 6 Hours | |
| | | ∑ | % | ∑ | % | ∑ | % | ∑ | % | ∑ | % |
| 10 | 10 | 0 | 0 | 6.6 | 66 | 7.4 | 74 | 8.2 | 82 | 8.6 | 86 |
| 15 | 10 | 0 | 0 | 6.4 | 64 | 6.6 | 66 | 7.6 | 76 | 7.6 | 76 |
| 20 | 10 | 0 | 0 | 4.2 | 42 | 4.6 | 46 | 6.6 | 66 | 6.6 | 66 |
| Control (-) | 10 | 0 | 0 | 7 | 70 | 7.6 | 76 | 7.8 | 78 | 8.4 | 84 |
| Control (+) | 10 | 0 | 0 | 0 | 0 | 0.8 | 8 | 1.4 | 14 | 1.6 | 16 |

The observation results shown in table 1 indicated a clear difference between the treatment groups and the control in the number of mosquitoes that landed. In general, higher extract concentrations demonstrated better repellent effectiveness across all observation time points. At hour 1, the 20% concentration showed the lowest mosquito landing rate (42%), compared to 15% (64%) and 10% (66%). This indicates that increasing the concentration improved the initial repellent effectiveness by approximately 24% compared to the lowest concentration. However, the effectiveness of all treatment groups declined over time. At hour 6, an increase in the number of mosquitoes landing was observed across all concentrations, at 86% (10%), 76% (15%), and 66% (20%). Compared to the initial condition at hour 1, the 20% concentration experienced a decline in protective effectiveness of approximately 24 percentage points, indicating a gradual reduction in the activity of the extract.

When comparing between treatments at hour 6, the 20% concentration still demonstrated higher effectiveness than 10% (a difference of 20%) and 15% (a difference of 10%). This indicates that increasing the concentration not only improved initial effectiveness, but also provided relatively better durability of effect, despite still experiencing a decline. Compared to the negative control, all extract treatments showed a lower number of mosquitoes landing at all observation time points, indicating the presence of repellent activity from the *Azolla microphylla* extract. Conversely, the positive control showed the lowest and most stable landing rate (0-16%), indicating more consistent repellent effectiveness of the synthetic repellent compared to the natural extract.

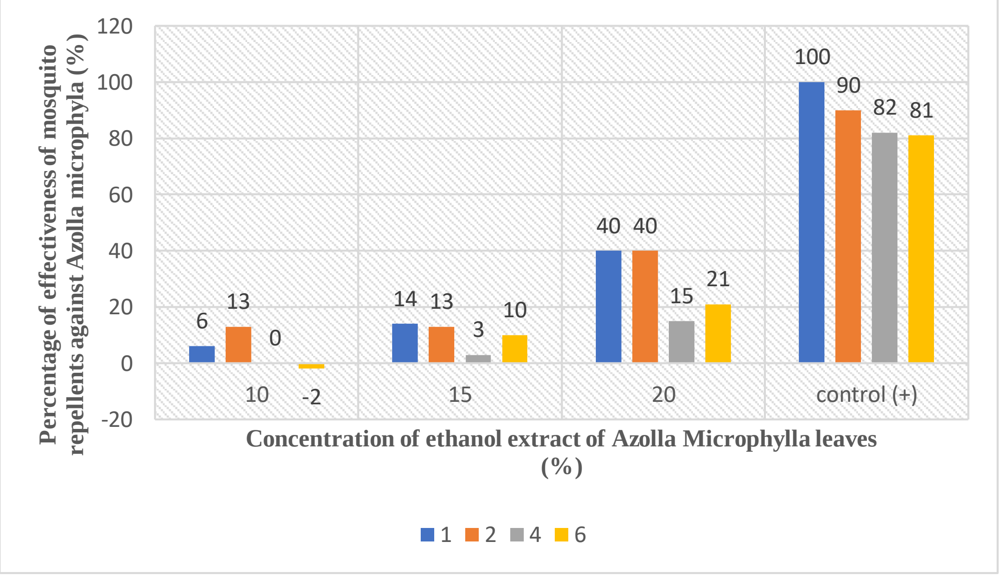


**Figure 1. Graphic Percentage of effectiveness of mosquito repellent power of ethanol extract of *Azolla microphylla* leaves**

The results depicted in figure 1 showed that ethanol extract of *Azolla microphylla* leaves at concentrations of 10%, 15%, and 20% decreased mosquito repellent activity at each observation time interval: at 1, 2, 4, and 6 hours. This decrease in effectiveness was also seen in the positive control, indicating that the repellent activity of a compound is unstable over time. There appears to be a greater increase in repellent potential at the beginning of exposure at a concentration of 20% *Azolla microphylla* leaf ethanol extract, but the effectiveness continues to decline gradually. This indicates that the *Azolla microphylla* extract does not have sufficient stability to maintain its repellent effect over a long period of time. Compared to synthetic repellents such as DEET (positive control), plant extracts tend to have a shorter duration of protection because their active compounds are not specifically formulated to slow evaporation.

The decrease in repellent activity in *Azolla microphylla* extract is attributed to the volatility of the active components contained in the leaves, such as flavonoids, tannins, and alkaloids. These compounds generally readily evaporate or degrade when exposed to air and ambient temperature, resulting in a decrease in the concentration of active ingredients remaining on the skin surface or in the test medium. As the concentration of active compounds decreases, the extract's ability to inhibit mosquitoes' approach also decreases (Ekawati & Pradana, 2019; Chanapanchai et al., 2025).

This statement is supported by statistical analysis, which showed that at 0 hours, the significance value was 1.000 (>0.05). This indicates that there was no significant difference between the *Azolla microphylla* extract treatment and the control at the beginning of the observation. In other words, in the initial minutes of application, the conditions of all groups were relatively similar, so the repellent effect was not statistically significant. However, at 1 hour to 6 hours, the significance value was 0.001 (<0.05) for each observation period. These results indicate a significant difference between the treatment and control groups at hours 1, 2, 4, and 6. This means that after 1 hour of exposure, the extract's repellent effectiveness began to show statistically significant changes.

This significant difference confirms previous findings that the repellent effectiveness of *Azolla microphylla* extract decreases with time. This may be due to the volatility of the active compound, which is volatile, resulting in a decrease in the remaining compound concentration, leading to a decrease in repellent activity (Suari et al., 2021). This condition also appears consistent with the trend in the positive control, which showed a similar pattern of decreasing effectiveness.

Beyond the general trend, a more detailed quantitative comparison highlights the magnitude of differences between treatments. At 1 hour of observation, the 20% concentration reduced the number of mosquitoes landing by 24 percentage points compared to the 10% concentration (42% vs. 66%), and by 22 percentage points compared to the 15% concentration (42% vs. 64%). This indicates a clear dose-dependent effect at the initial stage of exposure. However, these differences narrowed over time, where at 6 hours the gap between 20% and 10% remained at 20 percentage points (66% vs. 86%), while the difference between 20% and 15% decreased to 10 percentage points (66% vs. 76%). This indicates that although higher concentrations maintained better repellency, the relative differences in effectiveness between concentrations became less pronounced as time progressed.

When compared across time intervals, the 20% extract showed a decline in repellent performance of approximately 24 percentage points from 1 hour (42% mosquitoes landing) to 6 hours (66% mosquitoes landing). A similar trend was observed in the 15% and 10% treatments, indicating a time-dependent decline in repellent activity across all concentrations. Overall, the data indicate a concentration- and time-dependent pattern, in which higher concentrations provided stronger initial protection, but all treatments gradually lost effectiveness as exposure time increased.

The effectiveness of the repellent is directly related to the behavior of the mosquito that lands; its main function is to prevent the mosquito from landing on the skin and biting. Repellents do not always kill mosquitoes, but work by disrupting their sensory system. Mosquitoes possess various sensors, including chemical sensors, heat sensors, and color sensors. Based on the mosquito's sensors, natural ingredients have several repellent mechanisms, such as disrupting mosquito receptors, disrupting host-finding habits, masking the host's odor, causing irritation or toxicity, or a combination of these mechanisms (Kaur et al., 2022; Sharma et al., 2024).

Repellents work by disrupting the ability of mosquitoes to detect chemical signals originating from a host. Active compounds in plant extracts can mask or interfere with olfactory signals, thereby reducing the attraction of mosquitoes to treated areas. As a result, mosquitoes tend to avoid landing on surfaces containing repellent substances, which ultimately reduces the number of mosquitoes that land. This avoidance behavior is the primary indicator used in this study to evaluate repellent effectiveness (Sarni et al., 2023; Akbar et al., 2022).

Despite the demonstrated repellent activity of Azolla microphylla extract, the primary limitation of this study lies in the volatility of its active compounds, flavonoids, tannins, and terpenoids, which evaporate rapidly upon exposure to air and ambient temperature, resulting in a progressive decline in effectiveness over time. This instability renders the extract insufficient for prolonged protection compared to synthetic repellents such as DEET. To address this limitation, future development should explore advanced formulation strategies to slow the evaporation of active compounds and extend their protective duration. One promising approach is microencapsulation, a technique in which active compounds are enclosed within a polymeric shell that enables controlled, sustained release over time (Specos et al., 2010). Additionally, incorporation into emulsion-based carriers, nanoemulsions, or hydrogel matrices has been shown to improve both the stability and skin retention of plant-derived bioactive compounds (Nuchuchua et al., 2009). Combining these formulation technologies with optimized concentrations of Azolla microphylla extract could significantly enhance its practical applicability as a long-lasting, environmentally friendly alternative to chemical repellents. Future studies should therefore prioritize not only bioactivity testing but also formulation optimization to bridge the gap between laboratory efficacy and real-world application.

## 4. Conclusion

The ethanol extract of *Azolla microphylla* leaves demonstrated concentration-dependent repellent activity against *Aedes aegypti*, with the highest effectiveness observed at the 20% concentration during the initial exposure period. However, repellent activity declined progressively across all concentrations over 1-6 hours ($p < 0.05$), indicating insufficient stability of the active compounds for prolonged protection. These findings confirm that *Azolla microphylla* extract holds meaningful potential as a short-term, naturally derived repellent, yet its crude form remains limited for sustained use without further development. Future research should focus on formulation optimization, particularly encapsulation or the incorporation of fixative agents, to enhance the stability and durability of the active compounds, thereby improving the practical viability of *Azolla microphylla* as an environmentally friendly alternative to synthetic repellents.

Beyond the scope of this study, future investigations should also consider testing a broader range of concentrations, evaluating extract stability under varying environmental conditions (e.g., temperature and humidity), and conducting direct skin application tests to better approximate real-world efficacy. Comparative studies involving other plant-based repellents may further contextualize the relative potential of *Azolla microphylla*. Ultimately, the integration of optimized natural repellents into community-based Dengue Hemorrhagic Fever prevention programs could offer a safer, more sustainable complement to existing vector control strategies, particularly in regions where chemical repellent use poses health and environmental concerns.

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